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BIOCHEMICAL INDICATORS OF INFECTION AND INFLAMMATION IN BURN IN--ETC(U)  
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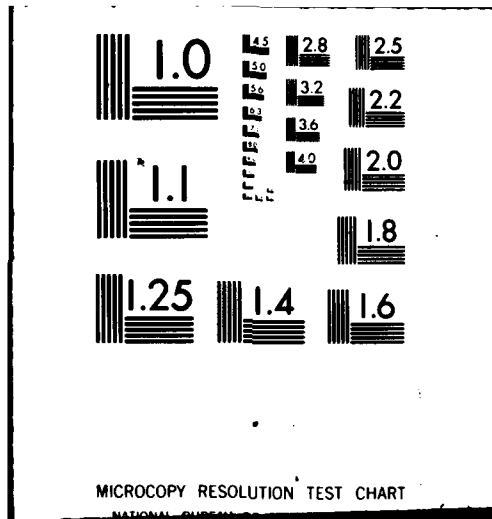
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BIOCHEMICAL INDICATORS OF INFECTION  
AND INFLAMMATION IN BURN INJURY (U)

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The treatment of severe thermal trauma is very often complicated by infection which occurs readily in such patients (1,2). The loss of the skin barrier and the extensive metabolic and physiologic alterations in burn patients renders detection of infection more difficult and may allow colonization to be mistaken for infection. Three factors have been found in perchloric acid filtrates of whole blood taken from burned-infected rats, two of which appear to be useful indicators of the presence of infection and the third a measure of the presence of inflammation, irrespective of etiology (3,4). The following presents the data that the 398 nm absorbance factor and the  $\lambda_{ex}$  355,  $\lambda_{em}$  420 fluorescent factor not only denote the presence of infection, but also reflect the severity of infection. Included is the evidence that these two factors require both plasma and cells for their generation as well as the attempts to ascertain which cell population is involved. The data from our initial studies to characterize the substances involved in the generation of these indicators are also presented.

METHODS AND MATERIALS

Rats used in these studies were obtained either from Holtzman or from Timco. The standard burn model of Walker and Mason (5) was used, and burn size varied as needed. Pseudomonas aeruginosa strain 12-4-4 was used to infect the burned rats. The biochemical indicators were quantitated as previously described (3) except that 0.2 ml of 30% H<sub>2</sub>O<sub>2</sub> was added prior to the measurement of the 355/420 fluorescent factor. Seromucoid was measured using a modification of the method of Chandler and Neuhaus (6). Partial correlation and multivariate regression was accomplished using the BMDP biomedical computer programs.

RESULTS AND DISCUSSION

The preparation of samples of whole blood for the determination of these biochemical indicators starts with the addition of cold perchloric

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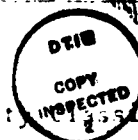
acid (PCA) to the whole blood; the final concentration of PCA is approximately 0.6 M. If plasma instead of whole blood were so treated, the resulting filtrate would contain the various carbohydrate-containing proteins which constitute the seromucoid fraction (7). Measurement of seromucoid concentration has been used to assess the severity of infection and/or injury (6,8,9,). Therefore, in some of the studies presented here, the concentration of seromucoid was also determined so as to provide an additional index of the severity of injury and/or infection. The biochemical indicators and seromucoid are all precipitated from their PCA filtrates by the addition of phosphotungstic acid (3,6) which suggests that the biochemical indicators may also be carbohydrate-containing proteins. There are a number of glycoproteins which are minor constituents of plasma; these proteins have not been extensively studied nor has their function been determined (9).

In order to assess the value of the newly discovered biochemical indicators of infection as compared to the seromucoid response to infection (7,8), the following study was carried out using fed and fasted rats. Twenty-four and 48 percent total body surface burns were produced, and one-half of each group of burned rats were inoculated with *Pseudomonas*. At 2 and 4 days, rats were killed and the blood analyzed for the biochemical indicators and the plasma for seromucoid. Figure 1 depicts the results of these analyses. On day 2, injury caused an increase in all indicators, while injury plus infection produced even greater changes. By day 4, the only significant increases in OD 398 and  $\lambda_{\text{ex}} 355/\lambda_{\text{em}} 420$  were the result of infection; there was little effect of injury. Seromucoid concentration as well as  $\lambda_{\text{ex}} 280/\lambda_{\text{em}} 340$  increased in response to injury alone, with somewhat greater changes due to injury plus infection. Fasting did not appreciably alter the extent of change in any of these indicators of infection and/or inflammation.

Table 1 summarizes the correlations between alterations in the indicators and burn size or the presence of infection. It is apparent that injury is a major factor in changes in concentration of these indicators on day 2, but on day 4, it is evident that the increases in OD 398 and fluorescence 355/420 are primarily in response to the presence of infection. This point is made clearer by Table 2, which shows the significance levels for the regression coefficients in Table 1. On day 4, OD 398 and fluorescence 355/420 do not respond significantly ( $P < 0.01$ ) to burn size, but do respond to the presence of infection.

In order to assess whether the biochemical indicators of infection reflected the severity as well as the presence of infection, groups of rats were vaccinated or inoculated with immune serum and then challenged with strain 12-4-4 *Pseudomonas*. Table 3 shows the effect of vaccination with a strain-specific ribosomal vaccine (10) on the generation of biochemical indicators. The vaccine protects > 90% of the burned-infected

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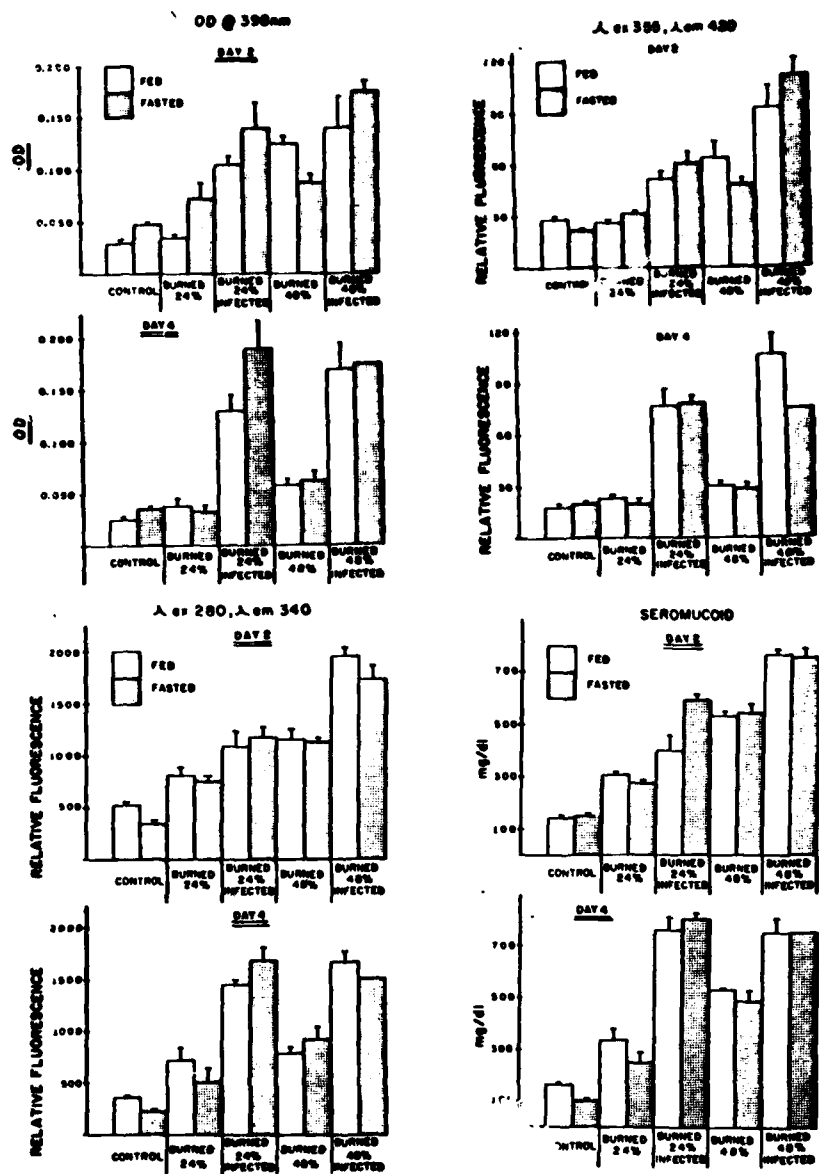


Figure 1. Alterations in putative biochemical indicators of infection and seromucoid following burn injury ± infection.

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Table 1. Correlation of Dependent Variables with Burn Size or Presence of Infection

	Day 2		Day 4	
	Burn Size	Infection	Burn Size	Infection
Seromucoid	.849	.655	.588	.844
OD 398	.621	.648	.304	.805
280/340	.793	.681	.493	.793
355/420	.689	.665	.334	.867

Table 2. Significance Levels for Regression Coefficients

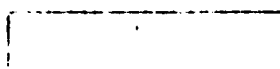
	Seromucoid	OD 398	280/340	355/420
Day 2: Burn size	.001	.001	.001	.001
Infection	.001	.001	.001	.001
Day 4: Burn size	.001	.070	.001	.014
Infection	.001	.001	.001	.001

Table 3. Effect of Vaccination with a Strain-Specific Ribosomal Vaccine on Generation of Biochemical Indicators

	Day 5				Day 7			
	OD 398	Fluorescence 280/340	Fluorescence 355/420	Sero-mucoid mg/dl	OD 398	Fluorescence 280/340	Fluorescence 355/420	Sero-mucoid mg/dl
Control	.147 ±.006	602 ± 20	40 ± 2	518 ± 25	.166 ±.015	482 ± 31	53 ± 5	498 ± 24
Burned-infected	.616 ±.050	1367 ± 51	229 ±19	1227 ± 96	.493 ±.068	1358 ±106	298 ±30	1102 ±102
Burned-infected once vaccinated day -7	.499 ±.109	1325 ±105	195 ±41	916 ± 81	.191 ±.010	1433 ±147	146 ± 1	902 ± 46
Burned-infected twice vaccinated days -7, -2	.334 ±.031	1350 ± 76	135 ±11	867 ± 56	.164 ±.030	1408 ± 94	147 ±24	946 ± 78

n = 6; mean ± SEM

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rats against death, while nonvaccinated burned-infected rats die between 7 and 10 days. Vaccination reduces the response of the 398 and 355/420 factors on day 5 and entirely abolishes the 398 response by day 7, while reducing the 355/420 response to 50% of the nonvaccinated rats. The 280/340 is unaffected by vaccination and seromucoid concentration only mildly suppressed. In contrast, treatment with a single injection of immune serum (11) which only lengthens the time to death but does not prevent it only slightly suppresses the production of all the indicators on day 4, but by day 7 there are no significant differences amongst the infected groups (Table 4).

Table 4. Effect of Treatment of Animals with Immune Serum on the Generation of Biochemical Indicators of Infection

	Day 4				Day 7			
	OD 398	Fluorescence 280/ 340	Sero- 355/ 430	mucoid mg/dl	OD 398	Fluorescence 280/ 340	Sero- 355/ 420	mucoid mg/dl
Control	.110 ±.008	617 ± 17	38 ± 2	483 ± 5	.121 ±.013	317 ± 60	28 ± 1	496 ± 19
Burned-infected	.539 ±.013	1667 ± 76	169 ±12	1152 ± 84	.564 ±.063	1367 ±117	177 ±10	1107 ± 55
Burned-infected pre-immune serum	.561 ±.054	1558 ± 84	198 ± 5	1147 ± 25	.503 ±.045	1617 ±106	162 ±11	1047 ± 46
Burned-infected immune serum	.407 ±.043	1375 ± 76	133 ±14	899 ± 56	.453 ±.072	1633 ±136	150 ±12	1067 ± 46

n = 6; mean ± SEM; sera given i.p. on day 0

Our initial attempts at purifying the biochemical indicators as they existed in acid filtrates of whole blood were frustrated by the lability of these factors under these conditions and their tendency to disappear when the filtrates were neutralized. This led us to re-examine the question of the localization of these factors, i.e., whether in plasma or cell associated, and the requirements for the generation of each of these indicators.

Table 5 shows that the OD factor exists neither in the plasma nor the cell fractions but only when plasma and cells are mixed together. This is essentially true also for the 355/420 fluorescent factor. In contrast, the 280/340 fluorescent factor is found in plasma but not in

cells, and in fact the addition of cells appears to interfere with its determination. Figure 2 displays the results of varying the concentration of plasma in the presence or absence of a constant amount of cells. The appearance of the OD 398 factor and the 355/420 fluorescent factor varies as a function of the amount of plasma present as long as cells are included. There is little 398 nm material detectable in the absence of cells, but there appears to be some 355/420 fluorescence even in the absence of cells. This may be due to factors released from the Pseudomonas in the infected animals. The 280/340 factor also varies as a function of plasma concentration, but the presence of cells serves to interfere with its detection.

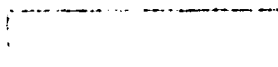
Table 5. Localization of Biochemical Indicators

Indicator	Sample	Group		
		Control	Burned	Burned-Infected
Absorbance 398 nm	Whole blood	.096 ± .002	.123 ± .012	.617 ± .137
	Plasma	0	0	.012 ± .003
	Cells	.074 ± .019	.053 ± .010	.050 ± .012
	Plasma + cells	.060 ± .005	.047 ± .010	.324 ± .045
Fluorescence 280/340	Whole blood	395 ± 48	453 ± 21	1325 ± 65
	Plasma	1288 ± 75	1233 ± 29	3950 ± 129
	Cells	127 ± 25	103 ± 6	206 ± 118
	Plasma + cells	670 ± 53	613 ± 32	1588 ± 103
Fluorescence 355/420	Whole blood	21 ± 3	28 ± 2	143 ± 18
	Plasma	8 ± 1	10 ± 5	28 ± 5
	Cells	8 ± 1	8 ± 2	10 ± 2
	Plasma + cells	56 ± 6	56 ± 6	90 ± 6

n = 4; mean ± SD

Table 6 clearly demonstrates that it is the source of plasma, not of cells, which is critical in the generation of the biochemical indicators. Plasma from control or burned rats when mixed with cells from burned-infected rats does not promote generation of indicators. However, plasma from burned-infected rats when mixed with cells even from control rats produces the 398 and 355/420 factors. The possible advantage of this finding is that retrospective analyses of stored plasma samples from animals (and, one hopes, patients) suspected of having been infected can be carried out using normal cells, perhaps even from a different species.

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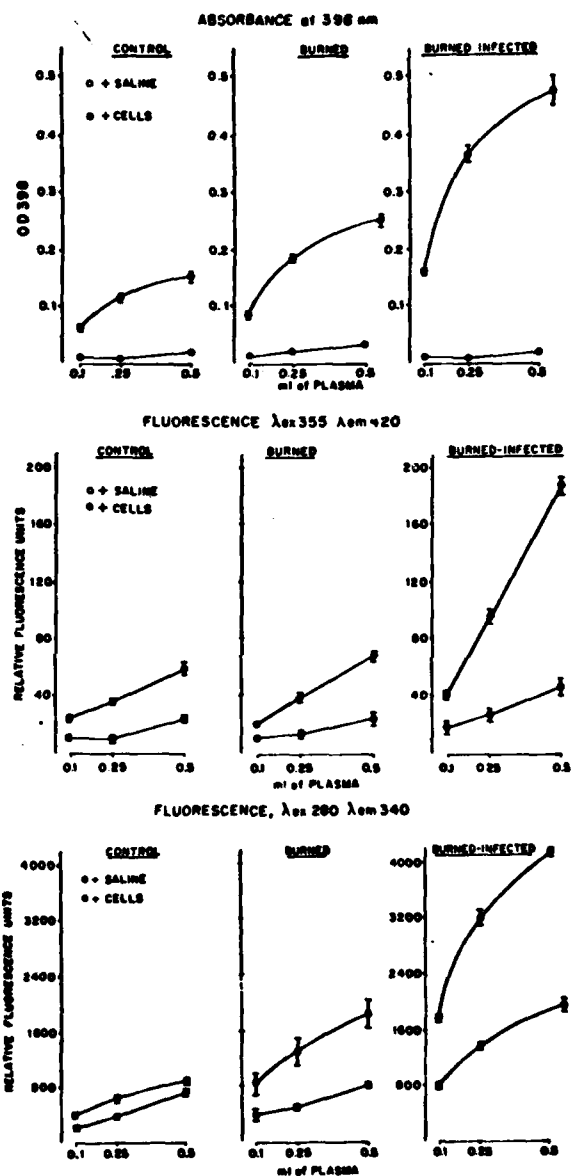


Figure 2. Titration curves for biochemical indicators of infection ± cells, cell concentration held constant.

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Table 6. Generation of Biochemical Indicators Dependent on Source of Plasma, not of Cells

	OD 398	Fluorescence 280/340	Fluorescence 355/420
Plasma control + cells burned-infected	.047 ± .003	773 ± 40	28 ± 3
Plasma burned + cells burned-infected	.039 ± .006	720 ± 15	28 ± 3
Plasma burned-infected + cells burned-infected	.234 ± .027	2400 ± 177	111 ± 4
Plasma burned-infected + cells control	.255 ± .019	2338 ± 197	114 ± 11
Plasma burned-infected + cells burned	.239 ± .011	2375 ± 109	125 ± 11

n = 4; mean ± SEM

To ascertain what population of cells were requisite for the generation of the 398 and 355/420 factors, Ficoll-hypaque gradients were employed. It would appear that the erythrocyte fraction (which also contains granulocytes in this preparation) is the required fraction (Table 7). To assess whether the few granulocytes admixed with the erythrocytes could be responsible for the generation of the 398 and 355/420 factors, a Percoll gradient was utilized. Granulocytes plus plasma do not result in the production of the 398 and 355/420 factors whereas erythrocytes plus plasma do (Table 8).

The discovery that erythrocytes were one of the components responsible for the generation of the 398 and 355/420 indicators of infection led us to test whether hemoglobin or other heme-containing substances could participate in the generation of these indicators. Table 9 demonstrates that hemoglobin, methemoglobin, myoglobin and even hemin can all interact with plasma from burned-infected animals to generate the 398 nm indicator. Except for methemoglobin, all of these compounds can participate equally well in the production of the 355/420 fluorescent indicator. The inability of methemoglobin to generate appreciable quantities of 355/420 material does not seem to be due to the presence of iron in the ferric form since this is also true of hemin. None of the compounds, except for hemoglobin, generates much of the 398 nm or 355/420

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Table 7. Ficoll Gradient Separation of Cells Responsible for Generation of Biochemical Indicators of Infection

	OD 398	Fluorescence 280/340	Fluorescence 355/420
Plasma + saline	.039 ± .006	3112 ± 123	46 ± 3
Plasma + cells	.199 ± .023	1838 ± 131	116 ± 4
Plasma + supernatant	.011 ± .003	1200 ± 20	37 ± 2
Plasma + lymphocyte/ monocyte band	.036 ± .010	52 ± 2	49 ± 4
Plasma + RBC pellet	.338 ± .014	35 ± 14	108 ± 3

n = 4; mean ± SEM

Table 8. Percoll Gradient Separation of Cells Responsible for Generation of Biochemical Indicators of Infection

Sample	OD 398 nm	Fluorescence	
		280/340	355/420
Whole blood	559 ± .029	878 ± 21	118 ± 2
Plasma + packed cells	.463 ± .022	990 ± 35	89 ± 4
Plasma + erythrocytes	.399 ± .017	910 ± 31	81 ± 1
Plasma + monocytes/ lymphocytes	.018 ± .002	2667 ± 133	31 ± 1
Plasma + granulocytes	.020 ± .004	2100 ± 106	28 ± 1
Plasma + saline	.026 ± .003	3700 ± 68	26 ± 1

n = 6; mean ± SEM

Table 9. The Potential Role of Heme-containing Compounds in the Generation of Two of the Biochemical Indicators of Infection

Source of Ligand	+ Plasma		+ Saline	
	OD 398	355/420	OD 398	355/420
Cells	.745 $\pm$ .021	169 $\pm$ 1	.062 $\pm$ .004	7.3 $\pm$ 0.5
Hemoglobin	.580 $\pm$ .013	119 $\pm$ 4	.066 $\pm$ .010	36 $\pm$ 3
Methemoglobin	.495 $\pm$ .009	32 $\pm$ 1	.093 $\pm$ .003	6.3 $\pm$ 0.9
Myoglobin	.330 $\pm$ .011	145 $\pm$ 2	.043 $\pm$ .003	6.0 $\pm$ 0.4
Hemin	.819 $\pm$ .014	140 $\pm$ 2	.056 $\pm$ .005	1.8 $\pm$ 0.3

n = 4; mean  $\pm$  SEM

Additions of ligands (other than cells) were 0.5 ml of a 2.3 mM solution

indicators when mixed with saline instead of plasma and even hemoglobin only produces some 355/420 fluorescence but little or no 398 absorbance. Though it appears that heme-containing compounds could substitute for erythrocytes in the assay of plasma for the 398 nm and the 355/420 indicators, it seemed advisable to continue to use erythrocytes until the plasma components had been purified and identified to eliminate the possibility of spurious results.

The findings that the source of plasma was the critical factor in the generation of the 398 and 355/420 indicators and that erythrocytes appear to be the cells which interact with the plasma substances in the presence of PCA to produce these indicators allowed us to pursue the following approach to the characterization and identification of the indicators. Rather than having to work with an unstable acid filtrate of whole blood, we could use plasma from burned-infected animals and employ classical techniques for the purification of proteins. The samples resulting from these procedures could then be assayed for the presence of the indicators by adding erythrocytes from normal animals followed by PCA. We first tried selective heat denaturation followed by ammonium sulfate fractionation. We found we could heat the plasma at 60° C for 30 minutes with no loss of activity (Table 10), but with about a 30% decrease in total protein content. The fact that the indicators were resistant to this treatment indicated that complement, which is inactivated under these conditions, is unlikely to be involved in the generation of the indicators. The heat treated plasma was then

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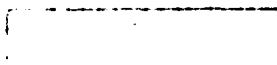


Table 10.  $(\text{NH}_4)_2\text{SO}_4$  Fractionation of Biochemical Indicators of Infection

Sample	OD 398 nm	Fluorescence	
		280/340	355/420
Untreated plasma	.157	3700	80
	.167	3700	81
60°, 30' plasma	.157	3650	82
	.160	3700	84
0-20% $(\text{NH}_4)_2\text{SO}_4$	.024	320	9
	.027	350	11
	.025	300	8
20-40% $(\text{NH}_4)_2\text{SO}_4$	.110	2450	36
	.118	2500	38
	.119	2475	38
40-60% $(\text{NH}_4)_2\text{SO}_4$	.529	2700	355
	.512	2600	360
	.500	2650	350
60-80% $(\text{NH}_4)_2\text{SO}_4$	.223	4950	73
	.204	5000	71
	.199	5000	73
Remainder	.021	250	9
	.023	270	8
	.022	280	10

subjected to ammonium sulfate fractionation. The preponderance of 398 and 355/420 generating factors could be found in the 40-60% saturation range, with some tailing into the 60-80% fraction (Table 10). In contrast, fluorescence 280/340 was polydisperse, with the greatest amount being found in the 60-80% fraction but with considerable such fluorescence detectable in the 20-40% and 40-60% fractions. The polydisperse nature of the 280/340 fluorescence is consistent with such fluorescence resulting from the presence of tryptophan in the protein and the fact that most proteins contain tryptophan. Thus it would appear that changes in 280/340 that occur in response to injury as well as infection reflect changes in the concentration of more than a single protein.

Since the 40-60% ammonium sulfate fraction contained the preponderance of the 398 and 355/420 factors, an aliquot of this fraction was applied to a Sephacryl S-200 column in an attempt to further purify the factors responsible for the generation of these two indicators and refine our estimate of their molecular weight (Figure 3). Column chromatography revealed that the factors responsible for the generation of all three indicators appeared to have in common a component (or components) which eluted as a single peak. Using serum albumin, MW 66,300 daltons, to standardize the column, one could estimate that the majority of proteins involved in the generation of the indicators had an apparent MW of 70-75,000 daltons. There were, however, other fractions from the column which contained 280/340 fluorescence associated with molecules considerably larger than albumin; this too is consistent with the idea that increases in 280/340 fluorescence during inflammation reflect increases in a number of proteins. When erythrocytes were used to assay column fractions for the presence of the factor(s) involved in the generation of the 398 nm indicator, there was some activity found in the area of the chromatograph which contained substances of molecular weights considerably less than 12,000 daltons. Perhaps this represents degradation products of the primary component in the 70-75,000 dalton fraction. If hemoglobin were substituted for erythrocytes in the assay, then the 398 factor displayed considerable heterogeneity with evidence for proteins of MW greater than 70-75,000 daltons being capable of generating the 398 factor.

At present we are continuing our efforts to purify, characterize and identify the plasma and cellular components which interact to generate the 398 and 355/420 indicators of infection. The available evidence indicates that these indicators are generated by the host in response to infection and not by the microorganism. One component of these indicators clearly is derived from erythrocytes, but the source of the plasma-borne component remains to be discovered. The plasma component could be synthesized de novo or merely in increased amounts by the liver as is the case for acute-phase proteins (12). Alternately the plasma component could be produced or released by cells of the reticuloendothelial system (13). Conceivably a substance already present in plasma could undergo transformation so as to be able to react with erythrocytes and generate a product which is soluble in 0.6 M perchloric acid. Another potential source for the plasma-borne component might be damaged cells. If damaged cells were a (rather than the) source of the plasma-borne component, this would explain why these indicators initially respond in some degree to injury but would not explain why these indicators rise dramatically in response to infection. Infection may elicit some tissue damage but certainly not to the extent that a 25% or 50% full-thickness burn would.

A preliminary survey of blood samples from burn patients indicates that some of these samples from very seriously ill patients appear to contain factors akin to those found in blood samples from burned-infected

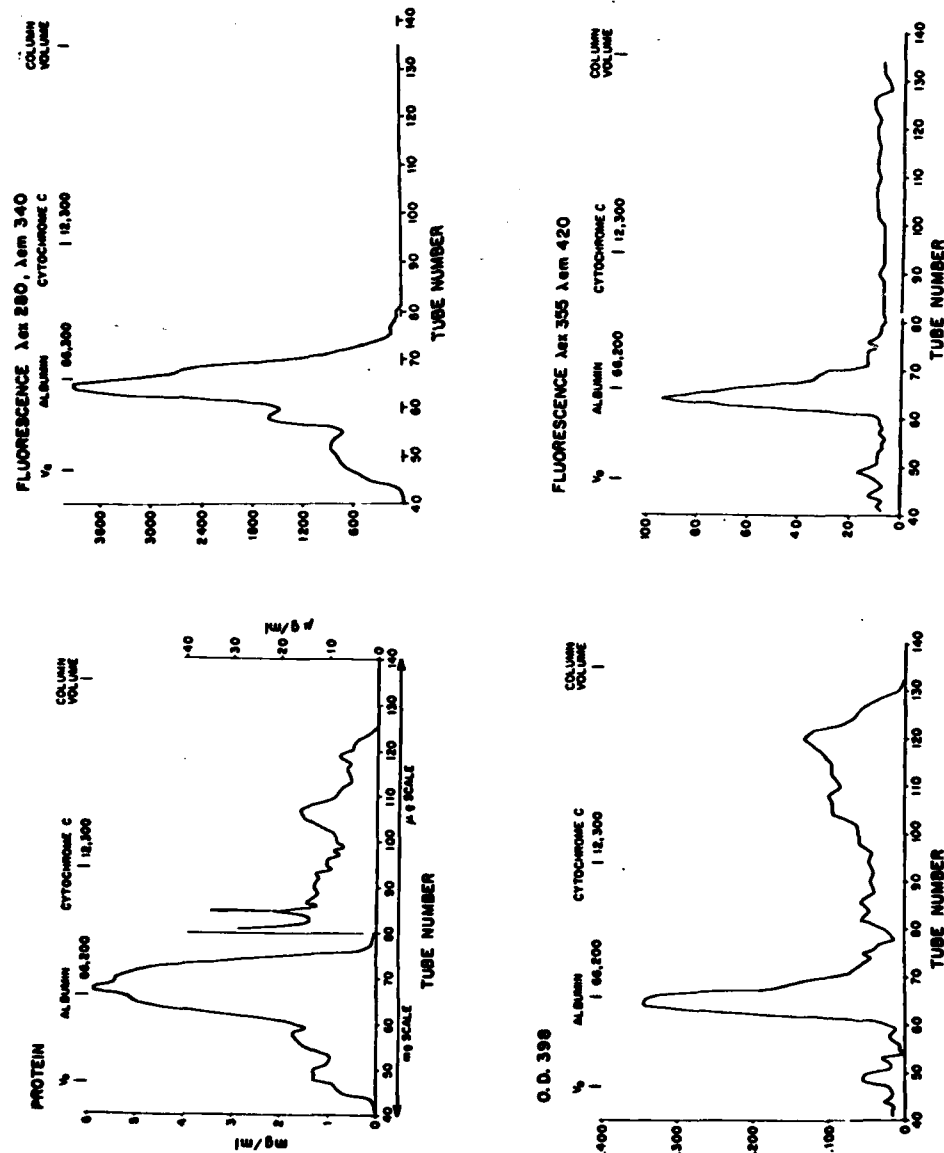


Figure 3. Separation of plasma-borne components of the biochemical indicators of infection on a Sephacryl S-200 column.

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animals. A study is under way to assess whether these factors are truly indicative of infection in these patients or merely provide additional, but quantitative, evidence of the severity of the patient's condition.

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